Form PTO-1390 (REV 10-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
1909.0030002	
U.S. APPLICATION NO (IF KNOWN, SEE 37 C.F.R. § 1.5)	
To be assigned 09/830964	
INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE
PCT/US99/26241	8 November 1999
PRIORITY DATE CLAIMED	
November 6, 1998	

TITLE OF INVENTION

Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants

APPLICANT(S) FOR DO/EO/US

Carlos Miguel Carcagno, Marcelo Criscuolo, Carlos Melo, and Juan Alejandro Vidal

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 372(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:
    Application Data Sheet
    Copy of International Search Report for PCT/US99/26240 (3 pages)

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
To be assigned 09/830964	PCT/US99/26241	1909.0030002

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... **$ 100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 690.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	16- 20 =	0	X **$18.00**	$ 0.00	
Independent Claims	1- 3 =	0	X **$80.00**	$ 0.00	
Multiple dependent claim(s) (if applicable)			+ **$270.00**	$ 0.00	
			TOTAL OF ABOVE CALCULATIONS =	$ 0.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$	
			SUBTOTAL =	$820.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$ 0.00	
			TOTAL NATIONAL FEE =	$ 820.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property			+	$	
			TOTAL FEES ENCLOSED =	$ 820.00	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $820.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
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SIGNATURE

Jorge A. Goldstein
NAME

29,021
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Carcagno *et al.*	Art Unit: *To Be Assigned*
Appl. No. 09/830,964	Examiner: *To Be Assigned*
Filed: May 3, 2001	Atty. Docket: 1909.0030002/JAG/CMB
For: **Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants**	

Preliminary Amendment and Submission of Sequence Listing

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notice to File Missing Parts of Application, dated June 5, 2001, in the above identified matter, and in advance of prosecution, please amend the application as follows:

In the Specification:

Please insert the sequence listing at the end of the application.

Remarks

No new matter has been added. The specification has been amended to direct the entry of this sequence listing after the claims of the above identified application.

In accordance with 37 C.F.R. § 1.821(g), this submission includes no new matter.

In accordance with 37 C.F.R. § 1.821(f), the paper copy of the Sequence Listing and the computer readable copy of the Sequence Listing submitted herewith in the above application are the same.

Summary

It is respectfully believed that this application is now in condition for examination. Early notice to this effect is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Cynthia M. Bouchez
Agent for Applicants
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Date: November 5, 2001

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SKGF Rev. 1 1/26/01 mac

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Carcagno *et al.*	Art Unit: To be assigned
Appl. No. To be assigned (U.S. Nat'l Phase of PCT/US99/26241)	Examiner: To be assigned
Filed: Herewith (International Filing Date: 8 November 1999)	Atty. Docket: 1909.0030002/JAG/CMB
For: **Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants**	

Preliminary Amendment

Commissioner for Patents
Washington, D.C. 20231

Sir:

Before examining the above application, please amend the application as follows.

It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Kindly enter the following amendment:

Amendments

In the Specification:

Please amend the specification as follows:

On the first page of the specification, on the line following the title, please insert the following --This Application claims benefit under 35 U.S.C. § 371 of International Application No. PCT/US99/26241, filed on 8 November 1999, which was published under PCT Article 21(2) in English and which is fully incorporated herein by reference.--

Remarks

Applicants respectfully request that this Preliminary Amendment be entered by the Examiner.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Jorge A. Goldstein
Attorney for Applicants
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Date: 5/3/01

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P:\USERS\Cbouchez\1909\0030002\prelim amend
SKGF Rev. 5/10/00

Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants

Background of the Invention

Field of the Invention

Methods to obtain recombinant human erythropoietin (EPO) characterized by a sequence of tandem separation steps that includes differential precipitation, hydrophobic interaction, anionic exchange, cationic exchange and molecular exclusion liquid chromatographies. The EPO obtained by using the methods thus described.

Background Information

EPO is a glycoprotein that stimulates erythroblast differentiation in the bone marrow, thus increasing the circulating blood erythrocyte count. The mean life of erythrocytes in humans is 120 days and therefore, a human being losses 1/120 erythrocytes each day. This loss must be continuously restored to maintain a stable level of red blood cells.

The existence of EPO was first postulated by the turn of the century and was definitely proved by Reissman and Erslev early in the '50s. See Carnot, et al., *C.R. Acad. Sci.* (France), 143, 384-6 (1906); Carnot, et al., *C.R. Acad. Sci.* (France), 143, 432-5 (1906); Carnot, et al., *C.R. Soc. Biol.*, 111, 344-6 (1906); Carnot, *C.R. Soc. Biol.*, 111, 463-5 (1906); Reissman, *Blood*, 1950, 5, 372-80 (1950) and Erslev, *Blood*, 8, 349-57 (1953). Reissman and Erslev's experiments were promptly confirmed by other researchers. See Hodgson, et al., *Blood*, 9, 299-309 (1954); Gordon, et al., *Proc. Soc. Exp. Biol. Med.*, 86, 255-8 (1954) and Borsook, et al., *Blood*, 9, 734-42 (1954).

The identification of the EPO production site in the organism was an issue of debate. Successive experiments led to identify the kidney as the main organ and peritubular interstitial cells as the synthesis site. See Jacobson, et al., *Nature*, 179, 633-4 (1957); Kuratowska, et al., *Blood*, 18, 527-34 (1961); Fisher, *Acta Hematol.*, 26, 224-32 (1961); Fisher, et al., *Nature*, 205, 611-2 (1965); Frenkel,

et al., *Ann. N.Y. Acad. Sci.*, 149, 1, 292-3 (1968); Busuttil, et al., *Proc. Soc. Exp. Biol. Med.*, 137, 1, 327-30 (1971); Busuttil, *Acta Haematol.*, (Switzerland), 47, 4, 238-42 (1972); Erslev, *Blood*, 44, 1, 77-85 (1974); Kazal, *Ann. Clin. Lab. Sci.*, 5, 2, 98-109 (1975); Sherwood, et al., *Endocrinology*. 99, 2, 504-10 (1976); Fisher, *Ann. Rev. Pharmacol. Toxicol.*, 28, 101-22 (1988); Jelkmann, et al., *Exp. Hematol.*, 11, 7, 581-8 (1983); Kurtz, et al., *Proc. Natl. Acad. Sci. (USA)*, 80, 13, 4008-11 (1983); Caro, et al., *J. Lab. Clin. Med.*, 103, 6, 922-31 (1984); Caro, et al., *Exp. Hematol.*, 12, 357 (1984); Schuster, et al., *Blood*, 70, 1, 316-8 (1986); Bondurant, et al., *Mol. Cell. Biol.*, 6, 7, 2731-3 (1986); Bondurant, et al., *Mol. Cell. Biol.*, 6, 7, 2731-3 (1986); Schuster, et al., *Blood*, 71, 2, 524-7 (1988); Koury, et al., *Blood*, 71, 2, 524-7 (1988); Lacombe, et al., *J. Clin. Invest.*, 81, 2, 620-3 (1988); Koury, et al., *Blood*, 74, 2, 645-51 (1989).

A smaller proportion, ranging from 10% to 15% of total EPO, is produced by the liver in adults. See Naughton, et al., *J. Surg. Oncol.*, 12, 3, 227-42 (1979); Liu, et al., *J. Surg. Oncol.*, 15, 2, 121-32 (1980); Dornfest, et al., *Ann. Clin. Lab. Sci.*, 11, 1, 37-46 (1981); Dinkelaar, et al., *Exp. Hematol.*, 9, 7, 796-803 (1981); Caro, et al., *Am. J. Physiol.*, 244, 5 (1983); Dornfest, et al., *J. Lab. Clin. Med.*, 102, 2, 274-85 (1983); Naughton, et al., *Ann. Clin. Lab. Sci.*, 13, 5, 432-8 (1983); Jacobs, et al., *Nature*, 313, 6005, 806-10 (1985); Erslev, et al., *Med. Oncol. Tumor. Pharmacother.*, 3, 3-4, 159-64 (1986). The EPO produced is directly proportional to the extent of tissular hypoxia and its expression rises by increasing the number of the EPO producing cells.

EPO has shown great efficiency in the treatment of anemia, especially anemia derived from renal failure. See Eschbach, et al., *N. England J. of Med.*, 316, 2, 73-78 (1987); Krane, *Henry Ford Hosp. Med. J.*, 31, 3, 177-181 (1983). Its therapeutical usefulness, however, has been limited due to the unavailability of a massive production method. The quantity and quality of the EPO obtained by the extractive systems known were insufficient. Recently, the use of recombinant DNA technology has made it possible to obtain large amounts of proteins. The application of these techniques to eukaryotic cells has allowed a large scale production of EPO. See patents US 5,688,679 (to Powell), US 5,547,933 (to Lin), US 5,756,349 (to Lin), US 4,703,008 (to Lin) and US 4,677,195 (to Hewick et al.).

Several techniques for the separation of glycoproteins such as EPO are currently available. Ultrafiltration, column electrofocusing, flat-bed electrofocusing, gel filtration, electrophoresis and isotachophoresis and some others chromatographic methods have been utilized for the purification of glycoproteins. The most widely used chromatographic techniques have been ionic exchange chromatography and adsorption chromatography.

The ionic exchange method is a separation technique by which the components of a solution are distinguished according to their different net charges and isolated by elution, either in stages or through the application of a continual gradient, with eluents of different ionic strength or pH. This method employs a gel or resin matrix, either of positive or negative charge, to induce binding or electrostatic adsorption of components with opposite charges. During desorption or elution, sample components are exchanged by ions present in the solution or buffer used to elute, or by a change in pH that alters the net charge of the molecule of interest.

Reverse phase adsorption chromatography involves separating the sample components according to their different polarities. Sample components are adsorbed through a resin composed of a silica matrix covered with an organic polymer by non-covalent bonding. The selective desorption of the components occurs afterwards by the elution with a non-polar solvent containing the eluent.

The separation techniques described above were utilized initially to separate relatively small hydrophobic or hydrophilic molecules. Their application to the purification of larger molecules, such as proteins, and specially complex proteins such as lipoproteins, nucleoproteins and glycoproteins, is more recent. Numerous publications illustrate the state of the art attained so far in protein separation.

See Soferet et al., "Handbook of Process Chromatography" (Academic Press Inc., San Diego, California, 1997); Olson, Ed., "Separation Technology" (Interpharm Press, Inc., Buffalo Grove, Illinois, 1995); Franks, Ed., "Protein Biotechnology" (Human Press, Totowa, New Jersey, 1993); Deutscher, Ed., "Guide to Protein Purification, Methods in Enzymology", Vol. 182, (Academic Press Inc., San Diego, California, 1991); Seetharam et al., Eds., "Purification and Analysis of Recombinant Proteins" (Marcel Dekker, Inc., New York, New York,

1991); Harria et al., Eds., "Protein Purification Applications" (Oxford University Press, Oxford, England, 1990); Brown, et al., *Analytical Biochemistry*, 99, 1-21, 1979; Harrison et al., "VDYAC TM Comprehensive Guide to Reverse Phase Materials for HPLC", pp. 1-12 (The Sep/A/Ra/Tions Groups, Hesperia, California, 1984). The use of monoclonal antibodies raised against the protein of interest is another known method of protein recovery.

Several specific methods for recombinant EPO separation have been recently reported. One of these methods consists in protein purification by anionic exchange chromatography with selective protease elimination, followed by reverse phase chromatography and filtration. See US patent 4,667,016 (to Lai et al.). This technique claims a yield of 16% EPO of unknown specific activity and purity.

Another method proposed for the separation of recombinant EPO consists in the application of reverse phase high pressure liquid chromatography (RP-HPLC) to a solution containing partially purified protein. See US patent 4,667,195 (to Hewick et al.). This method has been found irreproducible in practice. Morever, the non-polar solvents commonly employed or recommended for protein and polypeptide separation by means of RP-HPLC, include reagents such as acetonitrile, difficult to remove from the protein of interest and potentially toxic for human beings. See Parsons, et al., *Endocrinology*, 114, 6, 2223-7 (1984). It should be noted, however, that ethanol and formic acid aqueous solutions for protein elution have also been used. See Takagaki, et al., *Journal of Biological Chemistry*, 5, 4, 1536-41 (1980).

Even though there is abundant information regarding the production of recombinant human EPO, a purification method yielding EPO adequate for its utilization in human beings has not yet been described. A suitable protein purification method should yield EPO over 99% pure and free of contaminants such as: aggregated material, b) degraded material, c) spurious proteins and d) proteases. A protein purity under 99% or the presence of any of the above mentioned contaminants might be toxic for human beings.

On the other hand, many of the methods proposed for EPO purification are not efficient when applied to industrial scale protein production. The RP-HPLC method employs expensive organic solvents, which increases

purification costs. In addition, organic solvents are more difficult to handle and contaminant to the environment. Other purification methods proposed are irreproducible in practice or have a low yield.

Summary of the Invention

The novel method of the present invention describes, in detail, a system for EPO purification whereby a high recovery of a product of high purity and quality is achieved. This product may be used without further purification to formulate pharmaceutical compounds as injectable products for use in human medicine.

An advantage of the claimed invention is the attainment of EPO protease free without any undesirable molecular variants such as aggregates, degraded material or molecules of unexpected isoelectric point values. The EPO obtained by the claimed invention is over 99% pure and could be utilized to prepare pharmaceutical formulations adequate for administration to human beings without any additional purification step. The EPO obtained by the claimed invention is a micro-heterogeneous protein comprising between five to eight isoforms with isoelectric points ranging between 3.0 and 4.5 and *in vivo* specific biological activity over 100,000 IU/mg protein measured by a ^{59}Fe incorporation ex-hypoxic polycythemic mice assay and an EPO total mass spectophotometric assay at 280 nm.

An additional advantage of the claimed invention is its low environmental impact. The method claimed is a clean process that does not employ separation steps based on RP-HPLC technology, thus avoiding the use of organic solvents that may be harmful to the environment.

Yet another advantage of the claimed invention is the non-exposure of EPO to stringent temperature conditions, harmful organic solvents or other solutions that may affect its biological activity or result in a toxic compound unsuitable for human use.

The following detailed description and examples illustrate the separation steps performed in the claimed method.

Brief Description of the Figures

Figure 1 illustrates polyacrylamide gel (SDS-PAGE) analysis of an EPO sample obtained following the method described after purification. In lanes 1, 4 and 7, molecular weight markers were loaded. In lanes 2, 3, 5 and 6, different amounts of pure EPO obtained according to the claimed procedure were run. The purity of the product obtained and the apparent molecular weight exceeding 34 kDa is coincident with the one reported for urinary human EPO as could be clearly observed.

Figure 2 illustrates a Western blot analysis of an EPO sample obtained according to the method described. Identity of the EPO produced is assessed, since it is recognized by a monoclonal antibody against human EPO. In lanes 1 and 2, a human EPO standard and molecular weight markers were loaded, respectively. EPO samples obtained according to the claimed method were loaded in lanes 3 to 5.

Figure 3 shows a SDS-PAGE analysis of a pure EPO sample obtained according to the method described, treated with glycanases. Molecular weight markers were loaded in lanes 1, 4 and 8. Lanes 2 and 7 correspond to untreated EPO. In lane 3, O-glycanase treated EPO was loaded; the presence of an O-glycosilation site is verified. In lane 5, N-glycanase partially digested EPO was loaded. The presence of 3 N-glycosilated molecules with molecular weights as expected for EPO can be verified. Lane 6 was loaded with EPO digested with N-glycanase, and the expected molecular weight for the wholly deglycosilated protein was obtained.

Figure 4 illustrates a survey of the isoelectric points in pure EPO samples produced according to the method described. EPO samples were run in lanes 2, 3 and 4, isoelectric point markers in lanes 1 and 5. The presence of isoforms corresponding to EPO are verified, showing an isoelectric point range of 3.0 to 4.5.

Figure 5 shows the purity of an EPO sample produced according to the method herein described using a reverse phase high performance liquid chromatography.

Figure 6 illustrate the purity of an EPO sample produced according to the method herein described using a molecular exclusion high performance liquid chromatography.

Detailed Description of the Invention

The present invention relates to a method of purifying EPO which comprises treating cell culture supernatants comprising EPO by a combination of the following steps: differential precipitation, hydrophobic interaction chromatography, diafiltration, anionic exchange chromatography, cationic exchange chromatography and molecular exclusion chromatography.

Preferred EPO producing recombinant cells comprise a vector which comprises a nucleotide sequence encoding the EPO polypeptide consisting of the amino acid sequence in SEQ ID NO:1, a viral promoter and a viral terminator. Preferred cells contain vectors which confer resistance to both methotrexate and neomycin-derived antibiotics. Preferably, the EPO nucleic acid molecule comprises the nucleic acid molecule described in Lin, "DNA Sequences Encoding Erythropoietin," U.S. Patent No. 4,703,008. Preferably, the viral promoter is an SV40 early promoter.

A preferred method of obtaining EPO from recombinant cells is culturing in media comprising insulin. Specifically, such culturing comprises separating the supernatant which comprises EPO and insulin from the host cells of the invention, concentrating the supernatant and freezing the concentrated product. Preferably, the culture media comprises between 0.5 mg and 20 mg insulin per liter of culture media.

The invention further relates to a method of purifying EPO which comprises treating cell culture supernatants comprising EPO by the following steps in order: (a) differential precipitation, (b) hydrophobic interaction chromatography, (c) diafiltration, (d) anionic exchange chromatography, (e) cationic exchange chromatography and (f) molecular exclusion chromatography. The present invention relates to a method of purifying EPO which comprises treating cell culture supernatants comprising EPO by a combination of the

following steps: differential precipitation, hydrophobic interaction chromatography, 2 diafiltration steps, anionic exchange chromatography, cationic exchange chromatography and molecular exclusion chromatography.

The invention further relates to a method of purifying EPO which comprises treating cell culture supernatants comprising EPO by a following steps in order: (a) differential precipitation, (b) hydrophobic interaction chromatography, (c) diafiltration, (d) anionic exchange chromatography, (e) cationic exchange chromatography, (e') diafiltration and (f) molecular exclusion chromatography.

The differential precipitation step of the above and below-described methods and compositions comprises adding ammonium sulfate to said supernatant, followed by centrifugation.

The hydrophobic interaction chromatography step of the above and below-described methods and compositions comprises using an hydrophobic interaction matrix. Preferably said interaction matrix is Phenyl Sepharose 6 Fast Flow.

The anion exchange step of the above and below-described methods and compositions comprises using an anion exchange matrix. Preferably said anion exchange matrix comprises Q-Sepharose Fast Flow.

The cation exchange step of the above and below-described methods and compositions comprises using a cation exchange matrix. Preferably said cation exchange matrix comprises SP-Sepharose Fast Flow.

The molecular exclusion step of the above and below-described methods and compositions comprises using a molecular exclusion matrix. Preferably said molecular exclusion matrix is Sephacryl S-200 HP.

In another embodiment, the present invention provides a substantially pure EPO. Preferably, said EPO is produced by a combination of the following steps: differential precipitation, hydrophobic interaction chromatography, diafiltration, anionic exchange chromatography, cationic exchange chromatography and molecular exclusion chromatography. More preferably, said EPO has a purity of greater than 99% as determined by SDS-PAGE gel electrophoresis.

A preferred method of using the purified EPO of the present invention comprises lyophilization into a form suitable for injection into humans for

treatment of disease. Specifically, the preferred lyophilization procedure comprises placing the EPO into a pharmaceutical composition, loading the first EPO composition into a container, wherein said container is at a temperature equal to or less than -30°C; incubating said EPO composition at a temperature equal to or less than -30°C under atmospheric pressure for a time equal to or greater than 4 hours; incubating said composition at a pressure of equal to or less than 30 absolute microns for a time equal to or greater than one hour; and raising the temperature equal to or less than 3°C per hour until reaching at least 25°C, while keeping pressure values equal to or less than 5 absolute microns.

A preferred pharmaceutical composition for lyophilization comprises EPO, sugar, salts and human albumin. An especially preferred composition for lyophilization comprises EPO, mannitol, NaCl, NaH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$.

The present invention is described in further detail in the following non-limiting examples.

Examples

Example 1 Recovery

7,920 g of ammonium sulfate were dissolved in 30 liters of sterile concentrated solution obtained from culturing CHO (Chinese Hamster Ovary) cells producing EPO. After addition of ammonium sulfate, the solution was stored at 4 °C for 24 hours. Many contaminant proteins precipitated while the EPO remained in solution. The product was centrifuged at 5,000 RPM for 10 minutes in a Sorvall centrifuge, using a HG4L rotor.

Example 2 Hydrophobic Interaction Chromatography

The material obtained from the previous step is chromatographed using an Hydrophobic Interaction matrix (Phenyl Sepharose 6 Fast Flow low sub.-Pharmacia) according to the following parameters:

1. Equipment:

A. Pre-column:

1) Diameter: 14 cm

2) Bed height: 19 cm

3) Matrix:

a) Q-Sepharose Big Bead (Pharmacia)

b) Volume: 3,000 ml

B. Column:

1) Diameter: 20 cm

2) Bed height: 19 cm

3) Matrix:

a) Phenyl-Sepharose 6 Fast Flow low sub. (Pharmacia)

b) Volume: 6,000 ml

2. Solutions and buffers:

A. Buffer A: 10mM NaH_2PO_4, pH 7.2

B. Buffer F: 10mM NaH_2PO_4, 1.8 M $(NH_4)_2SO_4$, pH 7.2

C. Buffer G: 150mM NaH_2PO_4, pH 7.2

D. 20% isopropyl alcohol

E. 0.5 N NaOH

3. Material to be chromatographed:

A. Ammonium sulfate supernatant resulting from previous example.

B. Sample conditions:

1) Volume: 30,000 ml

2) Conductivity: 190-210 mSi/cm

3) pH: 7.2

To equilibrate and sanitize the pre-column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 volume of the column ("vc") (3 l) of H_2O; 1.0 vc (3 l) of NaOH 0.5N; 1.0 vc (3 l) of Buffer G and finally 1.5 vc (4.5 l) of Buffer F.

To equilibrate the column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 vc (6 l) of H_2O; 1.0 vc (6 l) of 20% isopropyl alcohol; 1.0 vc (6 l) of H_2O; 1.0 vc (6 l) of 0.5 N NaOH; 1.0 vc (6 l) of H_2O; 1.0 vc (6 l) of Buffer G and finally 1.5 cv (9 l) of Buffer F.

Once the pre-column and the column were equilibrated, the column was connected after the pre-column and the material to be chromatographed was loaded. Said loading was performed at 4°C, at a 19 cm/hour flow. Thereafter, the elution was performed at the same flow rate but at room temperature, and the solutions and buffers hereinafter detailed were passed through the columns in the following quantities and order: 2.5 vc (15 l) of Buffer F, (once this buffer has passed through, the pre-column was removed). Once the pre-column was removed, the chromatography was performed on the Phenyl Sepharose column on which a Buffer F-Buffer A gradient was applied starting from a 85:15 ratio of said buffers until 50:50 ratio of said buffers in a total volume of 10 vc (60 l) was reached.

When the gradient was finished, 1.5 vc (9 l) of Buffer F-Buffer A in a 30:70 ratio was passed through the column and finally 1.5 vc (9 l) of H_2O. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4°C.

Example 3 Concentration and Diafiltration

The fractions resulting from the previous example were concentrated and diafiltered according to the conditions described below:

1. Equipment:
   - A. Peristaltic pump: Watson Marlow - Cat. N° 302S
   - B. Tubing: Masterflex - Cat. N° 06402-18
   - C. Concentrator: Prep Scale Millipore CDU F006LC

2. Solutions and buffers:
   - A. 10mM Sodium Dodecyl Sulfate (SDS)

B. 1 mM Triton X-100

C. 0.1N NaOH

D. H_2O

E. Buffer A: 10 mM NaH_2PO_4, pH 7.2

3. Material to be processed:

A. Selected fractions resulting from the previous example.

B. Sample conditions:

1. Volume: 15,000-30,000 ml

2. Conductivity: 130-170 mSi/cm

3. pH: 7.2

The equipment was first cleaned, sanitized and equilibrated, and the following sequence of solutions and buffers were flowed through the equipment: 10 l of 10mM SDS; 40 l of H_2O; 10 l of 1 mM Triton X-100, 40 l of H_2O; 10 l of 0.1N NaOH; 40 l of H_2O and finally 5 l of Buffer A. The equipment was then ready to be used for concentration and diafiltration against Buffer A on the selected fractions, following the usual methodology.

The final volume of the concentrated product was between 2,000 to 3,000 ml, its conductivity was 1,100-1,550 μmSi/cm and its pH was 7.2.

Example 4 Anionic Exchange Chromatography

The material resulting from the previous example was chromatographed using an anionic exchange matrix, as follows:

1. Equipment:

A. Column:

1) Diameter: 14 cm

2) Bed height: 19 cm

3) Matrix

a) Q-Sepharose Fast Flow (Pharmacia)

b) Volume: 3,000 ml

2. Solutions and buffers:

A. Buffer A: 10mM NaH_2PO_4, pH 7.2

B. Buffer G: 150 mM NaH_2PO_4, pH 7.2

C. Buffer N: 50mM Acetic Acid, 500 mM NaCl, pH 4.0

D. Buffer S: 50mM Acetic Acid, pH 4.0

E. 0.5 N NaOH

3. Material to be chromatographed

A. Fractions selected from the hydrophobic interaction step, duly concentrated and diafiltered.

B. Sample conditions:

1) Volume: 2,000 to 3,000 ml

2) Conductivity: 1,100-1,550 μSi/cm

3) pH: 7.2

To equilibrate the column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 vc (3 l) of H_2O; 1.0 vc (3 l) of 0.5 N NaOH; 1.0 vc (3 l) of Buffer N; 2.0 vc (6 l) of Buffer S; 3.0 vc (9 l) of Buffer G; and finally 2.0 vc (6 l) of Buffer A.

Once the column was equilibrated, the material to be chromatographed was loaded. Said loading was performed at room temperature at 39 cm/hour. Thereafter, the elution was performed at the same flow rate and temperature, and the solutions and buffers hereinafter detailed were passed in the following order: 1.0 vc (3 l) of Buffer A and 4.0 vc (12 l) of Buffer S. Thereafter, a Buffer S-Buffer N step (50:50) in a total volume of 1.5 vc (4.5 l) was performed.

Once the step finished, 1.5 vc (4.5 l) of Buffer N was passed through the column. The selected EPO containing fractions, were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4°C.

Example 5 ***Cationic Exchange Chromatography***

The material resulting from the previous example was chromatographed using a cationic exchange matrix, as follows:

1. Equipment:

A. Column:

1) Diameter: 14 cm

2) Bed height: 19 cm

3) Matrix

a) SP-Sepharose Fast Flow (Pharmacia)

b) Volume: 3,000 ml

2. Solutions and buffers

A. Buffer D: 12.5 mM Na_2HPO_4, 4mM Citric acid. pH 6.0

B. Buffer E: 12.5 mM Na_2HPO_4, 4mM Citric acid, 0.5M NaCl, pH 6.0

C. 0.5 N NaOH

3. Material to be chromatographed

A. Fraction selected from the previous example adjusted to pH 6.0 with NaOH cc and diluted until reaching a conductivity of 4,800 μSi/cm (conductivity equal to Buffer D-Buffer E in a 93.5:6.5 ratio).

B. Sample conditions:

1) Volume: 5,000 ml

2) Conductivity: 4,800 μSi/cm (equal to Buffer D-Buffer E in a 93.5:6.5 ratio).

3) pH: 6.0

To equilibrate the column, the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 vc (3 l) of H_2O; 1.0 vc (3 l) of 0.5 N NaOH; 1.0 vc (3 l) of Buffer E and finally 1.5 vc (4.5 l) of Buffer D-Buffer E in a 93.5:6.5 ratio.

Once the column was equilibrated, the material to be chromatographed was loaded. Said loading was performed at room temperature at 39 cm/hour. Thereafter, the elution was performed at the same flow rate and temperature, and

the solutions and buffers hereinafter detailed were passed through it in the following order: 1.5 vc (4.5 l) of Buffer D-Buffer E in a 93.5:6.5 ratio. Thereafter, a gradient of Buffer D-Buffer E was applied starting from a 93.5:6.5 ratio of said buffers until a 50:50 ratio of said buffers in a total volume of 2.0 vc (6 l) was reached. Once the gradient was finished, 1.5 vc (4.5 l) of Buffer E was passed through the column. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4 °C.

Example 6 Concentration and Diafiltration

The fractions resulting from the previous example were concentrated and diafiltered according to the following parameters and conditions:

1. Equipment:
   - A. Peristaltic pump: Watson Marlow - Cat. N° 302S
   - B. Tubing: Masterflex - Cat. N° 06402-18
   - C. Concentrator: Prep Scale Millipore CDU F002LC
2. Solutions and buffers:
   - A. 10mM Sodium Dodecyl Sulfate (SDS)
   - B. 1 mM Triton X-100
   - C. 0.1N NaOH
   - D. H_2O
   - E. Buffer B: 10 mM NaH_2PO_4, 0.15 M NaCl, 0.05 mg/ml Lactose, pH 7.2
3. Material to be processed:
   - A. Selected fractions resulting from the previous step.
   - B. Sample conditions:
     1. Volume: 6,000 ml
     2. Conductivity: 5,000-8,000 μSi/cm
     3. pH: 6.0

The equipment was first, cleaned, sanitized and equilibrated, letting pass through it the following sequence of solutions and buffers: 10 l of 10 mM SDS; 40 l of H_2O; 10 l of 1 mM Triton X-100, 40 l of H_2O; 10 l of 0.1N NaOH; 40 l of H_2O and finally 5 l of Buffer B. In this way, the equipment was ready to be used for the concentration and diafiltration procedures against Buffer B on the selected fractions, following the usual methodology.

The final volume of the concentrated product was 350-600 ml, its conductivity was 15,500-19,000 mSi/cm, the pH was 7.2, and the solution was stored at 4°C.

Example 7 Molecular Exclusion Chromatography

The material resulting from the previous example was chromatographed using a molecular exclusion matrix, as follows:

1. Equipment:
   - A. Column:
     1) Diameter: 10 cm
     2) Bed height: 76 cm
     3) Matrix
        a) Sephacryl S-200 HP (Pharmacia)
        b) Volume: 6,000 ml

2. Solutions and buffers:
   - A. Buffer B: 10mM NaH_2PO_4, 0.15 M NaCl, 0.05 mg/ml Lactose, pH 7.2
   - B. 0.5 N NaOH

3. Material to be chromatographed
   - A. Fractions selected from the previous example, concentrated.
   - B. Sample conditions:
     1. Volume: 350 to 600 ml
     2. Conductivity: 15,500-19,000 μSi/cm

3. pH: 7.2

To equilibrate the column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 volume of the column ("vc") (6 l) of H_2O; 1.5 vc (9 l) of 0.5 N NaOH and finally 3.0 vc (18 l) of Buffer B. Once the column was equilibrated, 100 ml from the material to be chromatographed were loaded. Said loading was performed at room temperature at 27 cm/hour. Thereafter, the elution was performed at the same flow and temperature rates, and 0.75 vc (4.5 l) of Buffer B was passed through it. This procedure was repeated between four to six times, that is, until the material to be chromatographed was completely utilized. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4°C.

With this step the purification process was concluded. The EPO obtained has a purity degree superior to 99% and the entire purification process had a global yield of approximately 30%.

Example 8 EPO Assays

The EPO obtained in the previous example was assayed for identity and biological activity according to the following protocol.

In a denaturing SDS-PAGE gel the EPO was identified as a wide band of molecular weight as expected for EPO. See Figure 1. The band was recognized by monoclonal and polyclonal antibodies raised against human EPO in a Western blot assay as expected for EPO. See Figure 2. The treatment with glycanases proved the existence of the glycosidic chains in the extent and size as expected for EPO. See Figure 3. The EPO produced was shown to be composed of a series of species showing isoelectric points from 3.0 to 4.5 as expected for EPO. See Figure 4.

The complete amino acid sequence of the isolated protein, purified from the culture supernatant of transfected cell lines showed total homology with natural human erythropoietin whose 165 aminoacid sequence is as follows (SEQ ID NO:1):

NH_2--- Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp
Ser	Arg	Val	Leu	Glu	Arg	Tyr	Leu
Leu	Glu	Ala	Lys	Glu	Ala	Glu	***Asn***
Ile	Thr	Thr	Gly	Cys	Ala	Glu	His
Cys	Ser	Leu	Asn	Glu	***Asn***	Ile	Thr
Val	Pro	Asp	Thr	Lys	Val	Asn	Phe
Tyr	Ala	Trp	Lys	Arg	Met	Glu	Val
Gly	Gln	Gln	Ala	Val	Glu	Val	Trp
Gln	Gly	Leu	Ala	Leu	Leu	Ser	Glu
Ala	Val	Leu	Arg	Gly	Gln	Ala	Leu
Leu	Val	***Asn***	Ser	Ser	Gln	Pro	Trp
Glu	Pro	Leu	Gln	Leu	His	Val	Asp
Lys	Ala	Val	Ser	Gly	Leu	Arg	Ser
Leu	Thr	Thr	Leu	Leu	Arg	Ala	Leu
Gly	Ala	Gln	Lys	Glu	Ala	Ile	Ser
Pro	Pro	Asp	Ala	Ala	***Ser***	Ala	Ala
Pro	Leu	Arg	Thr	Ile	Thr	Ala	Asp
Thr	Phe	Arg	Lys	Leu	Phe	Arg	Val
Tyr	Ser	Asn	Phe	Leu	Arg	Gly	Lys
Leu	Lys	Leu	Tyr	Thr	Gly	Glu	Ala
Cys	Arg	Thr	Gly	Asp----COOH			

X glycosilation sites

The presence of the four glycosilation sites on the 165 amino acid chain, as well as the complex carbohydrate structure, and in particular, the sialic acid terminal residues, which characterize EPO were verified. These results were further supported by a biological activity assay of the produced protein by an ex-hypoxic polycythemic mice test, which showed complete concordance with the international EPO standard.

An EPO sample obtained according to the claimed method was submitted to a reverse phase and molecular exclusion high performance liquid chromatography analysis. In both cases, a purity over 99% was proved. See Figures 5 and 6.

The following table illustrates the recovery of each separation step corresponding to the claimed procedure.

STAGE	RECOVERY (%)
Cell Culture Supernatant	100
Hydrophobic Interaction Chromatography	70
Concentration and Diafiltration I	97
Anionic Exchange Chromatography	82
Cationic Exchange Chromatography	71
Concentration and Diafiltration II	95
Molecular Exclusion Chromatography	79

The following table illustrates the accumulated recovery of the purification sequence claimed in Claim 2.

STAGE	RECOVERY (%)
Cell Culture Supernatant	100
Hydrophobic Interaction Chromatography	70
Concentration and Diafiltration I	68
Anionic Exchange Chromatography	56
Cationic Exchange Chromatography	40
Concentration and Diafiltration II	38
Molecular Exclusion Chromatography	30

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

What Is Claimed Is:

1. A method of purifying recombinant human erythropoietin from cell culture supernatants comprising by a combination of the following steps:

(a) differential saline precipitation;

(b) hydrophobic interaction chromatography;

(c) concentration and diafiltration;

(d) anionic exchange chromatography;

(e) cationic exchange chromatography;

(f) concentration and diafiltration;

(g) molecular exclusion chromatography.

2. The method of Claim 1, wherein steps a) through g) are performed in the following order: (a), (b), (c), (d), (e), (f) and (g).

3. The method of Claim 1, wherein steps a) through g) are performed in the following order: (a), (c), (d), (e), (b), (f) and (g).

4. The method of Claim 1, wherein step a) comprises adding ammonium sulfate to said culture supernatant, followed by centrifugation.

5. The method of Claim 1, wherein step (b) comprises using a hydrophobic interaction matrix.

6. The method of Claim 5, wherein said hydrophobic interaction matrix employed is Phenyl Sepharose 6 Fast Flow.

7. The method of Claim 1, wherein step (d) comprises using an anionic exchange matrix.

8. The method of Claim 7, wherein said anionic exchange matrix is Q-Sepharose Fast Flow.

9. The method of Claim 1, wherein step (e) comprises using a cationic exchange matrix.

10. The method of Claim 9, wherein said cationic exchange matrix is SP-Sepharose Fast Flow.

11. The method of Claim 1, wherein step (g) comprises using a molecular exclusion matrix.

12. The method of Claim 11, wherein said molecular exclusion matrix employed is Sephacryl S-200 HP.

13. A substantially pure erythropoietin, produced according to the method of Claim 1.

14. The erythropoietin according to Claim 13, wherein said EPO has a purity exceeding 99% as determined by a polyacrilamide gel electrophoresis analysis (SDS-PAGE) and reverse phase and molecular exclusion liquid chromatography.

15. The erythropoietin according to Claim 13, wherein said EPO is characterized by a series of isoforms of isoelectric point values between 3.0 and 4.5.

16. The erythropoietin according to Claim 13, wherein said EPO shows homology to the amino acid sequence of SEQ ID NO:1.

FIG.1

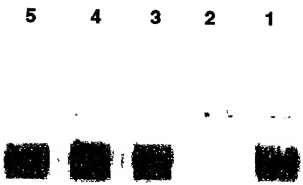

FIG.2

1
2
3
4
5
6
7
8
66,000
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14,000
66,000
36,000
24,000
18,000
14,000

FIG.3

FIG.4

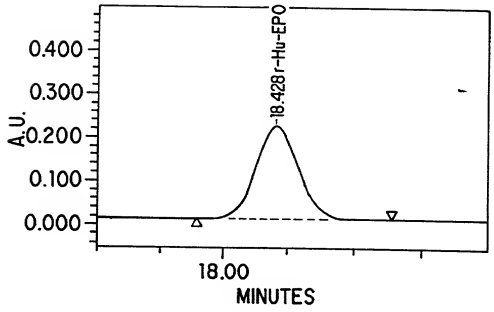

FIG. 5

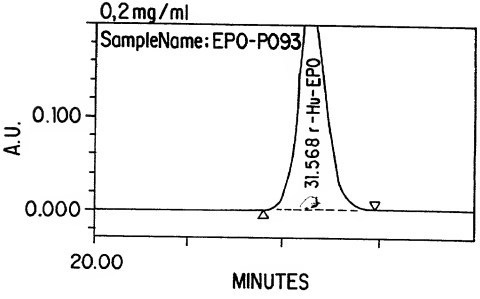

FIG. 6

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ASSIGNMENT

In consideration of the sum of One Dollar ($1.00) or equivalent and other good and valuable consideration paid to each of the undersigned inventors: Carlos M. Carcagno, Marcelo Criscuolo, Carlos Melo and Juan A. Vidal, the undersigned inventors hereby sell and assign to Sterrenbeld Biotechnologie North America, Inc. _____ (the Assignee) his/her entire right, title and interest, including the right to sue for past infringement and to collect for all past, present and future damages:

check applicable box(es) ☒ for the United States of America (as defined in 35 U.S.C. § 100),
☒ and throughout the world,

(a) in the invention(s) known as Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants for which application(s) for patent in the United States of America has (have) been executed by the undersigned on 1) Sept 19, 2001 2) Sept 11, 2001 3) Sept 4, 2001 4) Sept 24, 2001 also known as United States Application No. 09/830,964 , filed May 3, 2001), which is the U.S. National Phase of PCT/US99/26241, International Filing Date 8 November 1999 in any and all applications thereon, in any and all Letters Patent(s) therefor, and

$\frac{1}{24,021}$

(b) in any and all applications that claim the benefit of the patent application listed above in part (a), including continuing applications, reissues, extensions, renewals and reexaminations of the patent application or Letters Patent therefor listed above in part (a), to the full extent of the term or terms for which Letters Patents issue, and

(c) in any and all inventions described in the patent application listed above in part (a), and in any and all forms of intellectual and industrial property protection derivable from such patent application, and that are derivable from any and all continuing applications, reissues, extensions, renewals and reexaminations of such patent application, including, without limitation, patents, applications, utility models, inventor's certificates, and designs together with the right to file applications therefor; and including the right to claim the same priority rights from any previously filed applications under the International Agreement for the Protection of Industrial Property, or any other international agreement, or the domestic laws of the country in which any such application is filed, as may be applicable;

all such rights, title and interest to be held and enjoyed by the above-named Assignee, its successors, legal representatives and assigns to the same extent as all such rights, title and interest would have been held and enjoyed by the Assignor had this assignment and sale not been made.

The undersigned inventors agree to execute all papers necessary in connection with the application(s) and any continuing (continuation, divisional, or continuation-in-part), reissue, reexamination or corresponding application(s) thereof and also to execute separate assignments in connection with such application(s) as the Assignee may deem necessary or expedient.

The undersigned inventors agree to execute all papers necessary in connection with any interference or patent enforcement action (judicial or otherwise) related to the application(s) or any continuing (continuation, divisional, or continuation-in-part), reissue or reexamination application(s) thereof and to cooperate with the Assignee in every way possible in obtaining evidence and going forward with such interference or patent enforcement action.

The undersigned inventors hereby represent that they have full right to convey the entire interest herein assigned, and that they have not executed, and will not execute, any agreement in conflict therewith.

The undersigned inventors hereby grant Robert Greene Sterne, Esquire, Registration No. 28,912; Edward J. Kessler, Esquire, Registration No. 25,688; Jorge A. Goldstein, Esquire, Registration No. 29,021; David K.S.

Cornwell, Esquire, Registration No. 31,944; Robert W. Esmond, Esquire, Registration No. 32,893; Tracy-Gene G. Durkin, Esquire, Registration No. 32,831; Michele A. Cimbala, Esquire, Registration No. 33,851; Michael B. Ray, Esquire, Registration No. 33,997; Robert E. Sokohl, Esquire, Registration No. 36,013; Eric K. Steffe, Esquire, Registration No. 36,688; Michael Q. Lee, Esquire, Registration No. 35,239; Steven R. Ludwig, Esquire, Registration No. 36,203; John M. Covert, Esquire, Registration No. 38,759; and Linda E. Alcorn, Esquire, Registration No. 39,588; all of STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C., 1100 New York Avenue, N.W., Suite 600, Washington, D.C. 20005-3934, power to insert in this assignment any further identification that may be necessary or desirable in order to comply with the rules of the United States Patent and Trademark Office for recordation of this document.

IN WITNESS WHEREOF, executed by the undersigned inventors on the date opposite his/her name.

Date: ✔ 10/SEP/2001 Signature of Inventor: ✔
Carlos M. Carcamo

Date: ✔ 09/11/2001 Signature of Inventor: ✔
Marcelo Criscuolo

Date: ✔ 04/SEP/01 Signature of Inventor: ✔
Carlos Melo

Date: ✔ 09/24/01 Signature of Inventor: ✔
Juan A. Vidal

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POWER OF ATTORNEY FROM ASSIGNEE WITH DELEGATION

Sterrenbeld Biotechnologie North America, Inc., a corporation of Delaware, having a principal place of business at 1209mOrange Street, Wilmington, Delaware 19801, is assignee of the entire right, title, and interest for the United States of America (as defined in 35 U.S.C. §100), by reason of an Assignment to the Assignee executed on Sept 19, Sept 11, Sept 4, Sept 24, 2001, respectively of an invention known as Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants (Attorney Docket No. 1909.0030002/JAG/CMB), which is disclosed and claimed in a patent application of the same title by the inventors Carlos M. Carcagno, Marcelo Criscuolo, Carlos Melo and Juan A. Vidal (said application filed on May 3, 2001 at the U.S. Patent and Trademark Office, having Application Number 09/830,964).

29,04

The Assignee hereby appoint the U.S. attorneys associated with **CUSTOMER NUMBER 28393** to prosecute this application and any continuation, divisional, continuation-in-part, or reissue application thereof, and to transact all business in the U.S. Patent and Trademark Office connected therewith, such attorneys currently being: Robert Greene Sterne, Registration No. 28,912; Edward J. Kessler, Registration No. 25,688; Jorge A. Goldstein, Registration No. 29,021; David K.S. Cornwell, Registration No. 31,944; Robert W. Esmond, Registration No. 32,893; Tracy-Gene G. Durkin, Registration No. 32,831; Michele A. Cimbala, Registration No. 33,851; Michael B. Ray, Registration No. 33,997; Robert E. Sokohl, Registration No. 36,013; Eric K. Steffe, Registration No. 36,688; Michael Q. Lee, Registration No. 35,239; Steven R. Ludwig, Registration No. 36,203; John M. Covert, Registration No. 38,759; and Linda E. Alcorn, Registration No. 39,588. The Assignee hereby grants said attorneys the power to insert on this Power of Attorney any further identification that may be necessary or desirable in order to comply with the rules of the U.S. Patent and Trademark Office.

The Assignee hereby authorizes the U.S. attorneys named herein to accept and follow instructions from **Bio Sidus S.A.** as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and the Assignee. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by the Assignee.

Send correspondence to:

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Direct phone calls to 202-371-2600.

FOR: Sterrenbeld Biotechnologie North America, Inc.

SIGNATURE: ✔

BY: ✔ Marcelo L. Argüelles

TITLE: ✔ President

DATE: ✔ October 3, 2001

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Certificate Under 37 C.F.R. § 3.73(b)

Applicant: Carcagno *et al.*

Appl. No.: 09/830,964 (which is the U.S. National Phase of PCT/US99/26241) 35 U.S.C. § 371 Date: May 3, 2001

Entitled: Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants

Sterrenbeld Biotechnologie North America, Inc. (Name of Assignee), a corporation (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. [x] the assignee of the entire right, title, and interest, or

2. [ ] an assignee of an undivided part interest

in the patent application/patent identified above by virtue of either:

A. [x] An Assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the Patent and Trademark Office at Reel to be recorded, Frame to be recorded, or for which a copy thereof is attached.

OR

B. [ ] A chain of title from the inventor(s) of the patent application/patent identified above to the current assignee as shown below:

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[ ] Additional documents in the chain of title are listed on a supplemental sheet.

[x] Copies of assignments or other documents in the chain of title are attached.

[**NOTE:** A separate copy (*i.e.*, the original assignment document or a true copy of the original document) must be submitted to Assignment Division in accordance with 37 CFR Part 3, if the assignment is to be recorded in the records of the PTO. See MPEP 302-302.8]

The undersigned (whose title is supplied below) is empowered to act on behalf of the assignee.

Date: ✔ October 3, 2001

Name: ✔ Marcelo L. Argüelles

Title: ✔President

Signature: ✔

P:\USERS\Chouchen\1909\030002\1909003-2 cer
SKGF Rev 6/13/01 mac

Declaration for Patent Application

Docket Number: 1909.0030002/JAG/CMB

As a below named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled: Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants,

the specification of which is attached hereto unless the following box is checked:

☒ was filed on May 3, 2001 :
as United States Application Number 09/830,964 ; and
was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56, including for continuation-in-part applications, material information that became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or (f), or § 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or § 365(a) of any PCT international application, which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed	
P98 01 05610 (Application No.)	Argentina (Country)	6 November 1998 (Day/Month/Year Filed)	☒ Yes	□ No
P99 01 00680 (Application No.)	Argentina (Country)	23 February 1999 (Day/Month/Year Filed)	☒ Yes	□ No

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(202) 371-2600

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-

Full name of sole or first inventor
Carlos Miguel Carcagno

Signature of sole or first inventor
✔

Date
✔ 14/SEP/2001

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Citizenship
Argentina

Mailing Address
same as above

2-

Full name of second inventor
Marcelo Eduardo Criscuolo

Signature of second inventor
✔

Date
✔ 09/011/2001

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3-

Full name of third inventor
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Signature of third inventor
✔

Date
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SKGF Rev. 5/16/01 mac

(Supply similar information and signature for subsequent joint inventors, if any)

SEQUENCE LISTING

<110> Sterrenbeld Biotechnologie North America, Inc.
Carcagno, Carlos Miguel
Criscuolo, Marcelo
Melo, Carlos
Vidal, Juan Alejandro

<120> Methods of Purifying Recombinant Human
Erythropoietin from Cell Culture Supernatants

<130> 1909.0030002

<140>
<141>

<150> AR 99-01-00680
<151> 1999-02-23

<150> AR 98-01-05610
<151> 1998-11-06

<160> 1

<170> PatentIn Ver. 2.0

<210> 1
<211> 165
<212> PRT
<213> Homo sapiens

<400> 1

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Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe
         35                  40                  45
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Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp
     50                  55                  60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu
 65                  70                  75                  80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp
                 85                  90                  95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu
            100                 105                 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
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Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
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Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala
145                 150                 155                 160

Cys Arg Thr Gly Asp
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